

Mechanism of 5-fluorouracil required resistance in human hepatocellular carcinoma cell line Bel₇₄₀₂

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Abstract

AIM: To investigate the resistance mechanism of 5-fluorouracil (5-FU) in Bel₇₄₀₂/5-FU cells which was established in our lab by *in vitro* continuous stepwise exposure of human hepatocellular carcinoma (HCC) cell line Bel₇₄₀₂ to 5-FU.

METHODS: The expression of multidrug resistance-associated protein (MRP) and thymidylate synthase (TS) in Bel₇₄₀₂ cells was detected by immunocytochemistry. The fluorescein (FLU) accumulation, an index of MRP functional activity, was determined by flow cytometry. The distribution of FLU was observed by confocal laser scanning microscope. The spectrofluorometry was used to show the intracellular content of glutathione (GSH). Cell growth inhibition was determined by MTT assay. The activity of glutathione S-transferases (GSTs) was determined by spectrophotometry.

RESULTS: A higher expression of MRP in the Bel₇₄₀₂/5-FU cells was observed by using monoclonal mouse anti-MRP antibody, MRPr-1, in comparison with Bel₇₄₀₂ cells. Bel₇₄₀₂/5-FU cells also showed a significant decrease of FLU accumulation. FLU mainly accumulated in the nucleus with a high nuclear/cytoplasmic ratio in Bel₇₄₀₂ cells, whereas there was no difference of FLU accumulation between the nucleus and cytoplasm in Bel₇₄₀₂/5-FU cells. The intracellular GSH content in Bel₇₄₀₂/5-FU cells was almost 3 folds higher than that in Bel₇₄₀₂ cells. Addition of D, L-buthione-S, R-sulfoximine (BSO) dose-dependently reduced the GSH content in Bel₇₄₀₂/5-FU cells, however, only a weak enhancement on the cytotoxicity of 5-FU and doxorubicin (Dox) to Bel₇₄₀₂/5-FU cells was observed. Bel₇₄₀₂/5-FU cells also exhibited 29.1 % higher total GSTs activity than Bel₇₄₀₂ cells. Immunocytochemical staining by using anti-TS monoclonal antibody TS 106 showed that the level of TS in Bel₇₄₀₂/5-FU cells elevated markedly as compared with Bel₇₄₀₂ cells.

CONCLUSION: The continuous exposure of Bel₇₄₀₂ cells to 5-FU led to overexpression of TS and MRP, as well as increased intracellular GSH content and total GST activity.

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INTRODUCTION

It is well known that HCC is one of the malignant tumors with poor chemosensitivity to anticancer agents. To date, the fluoropyrimidine fluorouracil, such as 5-FU, is still the first choice drug in the treatment of HCC^[1-3]. However, its usage is limited because of the rapid development of acquired resistance. So far, there is no paper dealing with the establishment of acquired resistance of 5-FU in HCC and its active mechanism of resistance in the literature. Then we established a 160-fold 5-FU resistant cell line named Bel₇₄₀₂/5-FU from the parental HCC Bel₇₄₀₂ cell line through about 6 months of continuous 5-FU selection. In addition to resistance to 5-FU, the Bel₇₄₀₂/5-FU cells also showed 3-4-fold cross-resistance to Dox and cytarabine, but no cross-resistance to vincristine, taxol, cisplatin and hydroxycamptothecin. Our prior study revealed that Bel₇₄₀₂ and Bel₇₄₀₂/5-FU cells showed no difference in P-glycoprotein (P-gp) levels.

Nishiyama^[4] showed that the increased expression of MRP was closely related to 5-FU resistance in gastrointestinal cancer cell lines. MRP is also a member of the ABC superfamily of membrane transport proteins, which has certain sequence homology with P-gp. The drug-resistance pattern conferred by MRP is similar but not identical to that of P-gp^[5,6]. It is believed that MRP prefers to transport anionic drugs and neutral drugs conjugated to acidic ligands, such as GSH, glucuronate, or sulfate^[7,8]. GSH has been suggested as an important component of MRP-mediated MDR and drug transport^[9]. GSH can combine with anticancer drugs to form less toxic and more water soluble GSH conjugates, which can be exported from cells by MRP^[10,11]. The conjugation reaction is catalyzed by GSTs. The elevation of cellular GSH and the elevated expression and activity of GSTs are associated with the development of drug-resistance phenotype of tumor cells to alkylating compounds, including melphalan, cyclophosphamide, chlorambucil, nitrogen mustard, doxorubicin^[12-14].

TS catalyses the reductive methylation of deoxyuridine monophosphate to deoxythymidine monophosphate using 5, 10-methylene-tetrahydrofolate as a methyl donor co-substrate. This reaction provides the only de novo source of dTMP and is an essential step in DNA biosynthesis. TS is a critical target for fluoropyrimidine, including 5-FU. *In vitro* and *in vivo* studies have reported that increased TS protein levels and gene amplification and/or decreased inhibition of TS activity may be associated with the resistance to 5-FU^[15,16].

The purpose of the present study was to determine whether the change of MRP, GSH/GST and TS contributed to the 5-FU resistance observed in Bel₇₄₀₂/5-FU cells.

MATERIALS AND METHODS

Materials

Drugs and reagents 5-FU was purchased from Xudong Haipu Pharmaceutical Inc. (Shanghai, China). 1-chloro-2,4-dinitrobenzene (CDNB) and O-phthalaldehyde (OPT), GSH and 3-4,5-dimethyl-thiazol-2,5-diphenyl tetrazolium bromide

(MTT) was obtained from Sigma Chemical Co. (St. Louis, MO). Monoclonal mouse anti-MRP, MRP-r1 and FITC-labeled goat anti-mouse IgG were obtained from Zhong Shan Biotechnology Co., LTD (Beijing, China). Thymidylate synthase Ab-1 TS 106 was obtained from Neomarker (Fremont, CA). Fluorescein (FLU) was obtained from Amresco (Solon, Ohio). **Cell lines** The parental human hepatocellular carcinoma Bel₇₄₀₂ cells and the 5-FU selected drug-resistant Bel₇₄₀₂/5-FU cells were grown in RPIM1640 (GIBCO) media containing 10 % newborn calf serum, 100 unit/mL penicillin and 100 µg/mL streptomycin. Cells were kept at 37 °C in a 5 % CO₂-95 % air atmosphere, and passaged by 0.25 % trypsin plus 0.02 % EDTA treatment. Bel₇₄₀₂/5-FU cells were routinely maintained in the presence of 10 µg/mL 5-FU. Cells were however cultured in the absence of 5-FU for at least 3 days before their use for experiments.

Methods

Immunofluorescence assay of MRP expression Bel₇₄₀₂ and Bel₇₄₀₂/5-FU cells were seeded on to sterilized slides. After 24hr of incubation, cells were washed twice with cold PBS and fixed with 70 % methanol at -20 °C for 10 min. All subsequent procedures were done at room temperature. All slides were washed once with blocking solution (1 % bovine serum albumin/5 % normal goat serum/PBS), incubated in blocking solution with 0.1 % Tween 20 for 30 min, followed by incubation with MRPr1 which was diluted 1:30 in blocking solution with 0.1 % Tween 20 for 1 hour. Then slides were washed once with blocking solution and incubated with FITC-conjugated goat anti-mouse IgG which was diluted 1:50 in blocking solution at room temperature for 1 hour. Finally, Slides were examined under confocal laser scanning microscope (BIO-RAD MRC1024) for fluorescence intensities^[17].

FLU accumulation Assessment of MRP functional activity was carried out by measuring the intracellular accumulation of FLU^[18]. The confluent Bel₇₄₀₂ and Bel₇₄₀₂/5-FU monolayers were preincubated for 30 min at 37 °C in the culture medium, then exposed to 100 µmol·L⁻¹ FLU for 3hr at 37 °C. After washing for three times with ice-cold PBS, cells were rapidly collected and intracellular fluorescence of FLU was immediately measured with flow cytometer (Coulter, EPICS XL).

Cellular FLU distribution Bel₇₄₀₂ or Bel₇₄₀₂/5-FU cells were plated onto 6-multiwell plate (Coster) for examining FLU distribution by using confocal laser scanning microscopy. The cells were exposed to 100 µmol·L⁻¹ FLU for 3hr at 37 °C. After this incubation period, the loading solutions were removed and cells were washed for three times with ice-cold PBS containing 1 % bovine serum albumin and examined under confocal laser scanning microscope (MERIDIAN™ Ultima 212)^[19].

Measurement of cellular GSH content Bel₇₄₀₂/5-FU cells incubated with or without different concentrations of BSO for 24 hrs and Bel₇₄₀₂ cells were lysed by addition of 0.2 % Triton X-100 in PBS. Afterwards, 20 % HPO₃ was added to precipitate proteins. The cells were centrifuged at 4 °C at 10 000 r.p.m. for 10 min and the supernatant fraction was used for the measurement of GSH^[20]. Briefly, to 0.4 mL of the supernatant, 0.1 mL OPT (containing 0.1 mg OPT) and 2.5 mL PBS (0.1 mol·L⁻¹)-EDTA (5 mmol·L⁻¹) buffer (pH 8.0) were added. After thorough mixing and incubation at room temperature for 15 min, the solution was transferred to a quartz cuvette. Its fluorescence intensity at 420nm was determined with the excitation at 350nm by a fluorescence spectrophotometer (HITACHI, MPF-4).

Chemoresensitivity testing The effect of BSO on the cytotoxicity of Dox and 5-FU to Bel₇₄₀₂/5-FU cells was assayed by a MTT method^[21]. Briefly, Bel₇₄₀₂/5-FU cells were plated in 96-well plates at 3000 cells per well. After 24hr, various

concentrations of the test drugs were added in quadruplicate samples for each concentration. The cells were exposed to drugs for continuous 3 days. Then MTT (0.5 mg·mL⁻¹) was added and the plates were incubated at 37 °C in 5 % CO₂ for 4hr before 150 µL 100 % dimethyl sulfoxide was added to each well. The optical density of each well was determined by a microplate reader (Bio-Rad Model 450) at a wavelength of 570nm.

Total GST activity Bel₇₄₀₂ and Bel₇₄₀₂/5-FU cells were washed with PBS and resuspended in 200 µL TMS solution (50 mmol·L⁻¹ Tris, pH 7.5, 3 mmol·L⁻¹ MgCl₂, 200 mmol·L⁻¹ sucrose). After sonication at 4 °C (60sec), they were centrifuged at 10 000rpm for 25 min. To 10 µL cell supernatant, 2.9mL sodium phosphate buffer (0.1 mol·L⁻¹, pH 6.5) and 0.1mL reduced GSH were added. The reaction was carried out at room temperature by the addition of 30mM CDNB (in DMSO). Formation of S-2,4 dinitrophenyl glutathione was monitored spectrophotometrically by the increase in absorbance at 340nm ($\epsilon_{340}=9.6 \text{ mM}^{-1} \cdot \text{CM}^{-1}$). GST activity was expressed as nmol·min⁻¹·mg⁻¹ protein in the cells^[22].

TS expression Bel₇₄₀₂ and Bel₇₄₀₂/5FU cells in the exponential phase of growth were seeded onto sterile slides and incubated at 37 °C in 5 % CO₂ for 24hr. After washing with cold PBS, cells were fixed in cold acetone for 10 min. The cells were subsequently rinsed in PBS and incubate with normal goat serum (10 % in PBS) at room temperature for 30 min to reduce nonspecific binding. Cells were then incubated with TS 106 (1:50 dilution) at 37 °C for 2hr. After being washed with PBS, all slides were incubated with FITC-labeled goat anti-mouse IgG at 37 °C for 1hr. All slides were washed with PBS again and examined under confocal laser scanning microscope (BIO-RAD MRC1024)^[23].

RESULTS

Expression of MRP

Fluorescence immunostaining signal was weakly detectable in Bel₇₄₀₂ cells and a denser staining was found in plasma membrane of Bel₇₄₀₂/5-FU cells. The resistant Bel₇₄₀₂/5-FU cells (Figure 1B) showed an enhanced immunoreactivity to MRP compared to that of sensitive Bel₇₄₀₂ cells (Figure 1A), which indicated that the resistant cells contained elevated level of MRP.

FLU accumulation as an index of MRP functional activity

The previous result showed that Bel₇₄₀₂ and Bel₇₄₀₂/5-FU cells differed in MRP expression, then we performed the functional MRP assay to demonstrate if the established difference in the levels of MRP in the two cells were indicative for differences in the MRP activity. FLU is a well-recognized substrate of MRP but not of P-gp. The use of FLU and FLU analogs as probes for MRP functional activity has been reported by Huai-Yun *et al*^[24]. The results of flow cytometry showed that the accumulation of FLU in Bel₇₄₀₂ cells was almost two times higher than that in Bel₇₄₀₂/5-FU cells (Figure 2). In other word, MRP functional activity in the resistant Bel₇₄₀₂/5-FU cells was higher than that in the parental cells.

FLU redistribution

In drug-selected MRP-overexpressing cells, alterations in drug localization have been observed in addition to decreased accumulation^[25]. The intracellular distribution of FLU was directly examined by confocal laser scanning microscopy (Figure 3). When exposed to FLU for 3 hours, besides the difference in FLU accumulation between the two cell lines, different localization of FLU in both cell lines was also

observed. FLU mainly accumulated in the nucleus with a high nuclear/cytoplasmic ratio in Bel₇₄₀₂ cells (Figure 3A), whereas there was no difference of location between nucleus and cytoplasm in Bel₇₄₀₂/5-FU cells (Figure 3B).

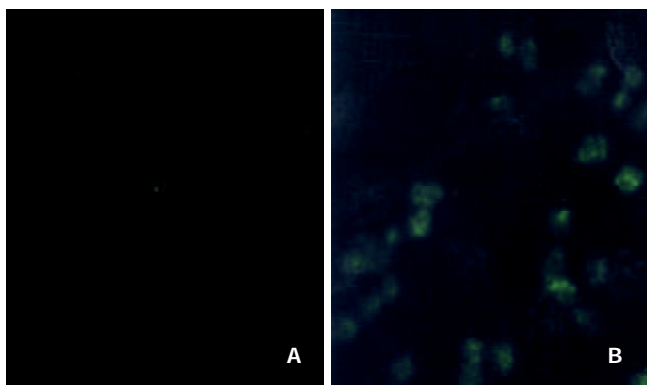


Figure 1 Immunocytochemical detection of MRP in Bel₇₄₀₂ and its 5-FU resistant cells in cytoprep slides. (A) Bel₇₄₀₂ cells; (B) Bel₇₄₀₂/5-FU cells. The cells were fixed in 70 % methanol, and incubated with anti-MRP monoclonal antibody MRPr-1 for 1hr at room temperature. FITC-conjugated goat anti-mouse IgG was added to examine the fluorescence staining of MRP under confocal laser scanning microscope

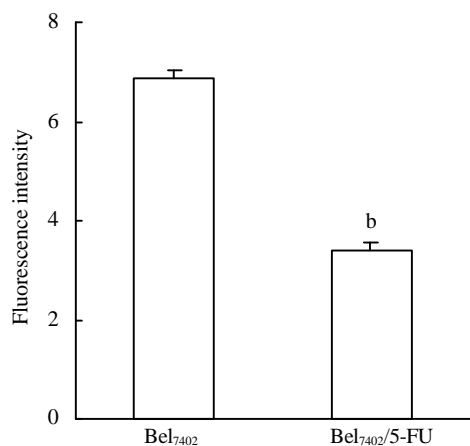


Figure 2 FLU accumulation in Bel₇₄₀₂ and Bel₇₄₀₂/5-FU cells. The cells were incubated with 100 $\mu\text{mol} \cdot \text{L}^{-1}$ FLU for 3hr. After washing, the cells were trypsinized and intracellular fluorescence of FLU was determined with a flow cytometer. Data were pooled from three independent experiments. ^b $P < 0.001$ vs Bel₇₄₀₂ cells

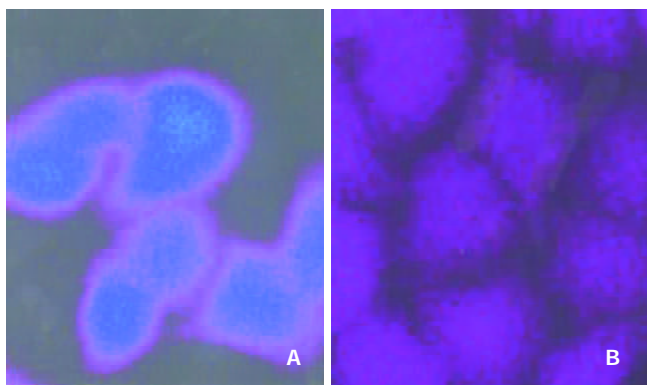


Figure 3 FLU distribution in Bel₇₄₀₂ and Bel₇₄₀₂/5-FU cells. The cells were incubated with 100 $\mu\text{mol} \cdot \text{L}^{-1}$ FLU for 3 hr. Then the intracellular distribution of FLU was revealed by confocal laser scanning microscopy. (A) Bel₇₄₀₂ cells; (B) Bel₇₄₀₂/5-FU cells.

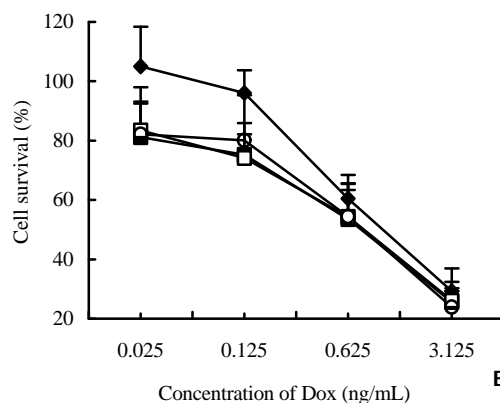
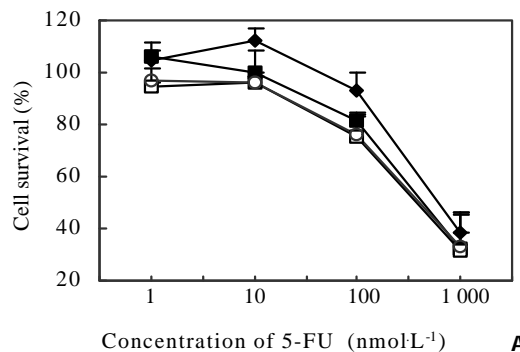


Figure 4 Effect of BSO on the cytotoxicity of 5-FU and Dox to Bel₇₄₀₂/5-FU cells. The cells were incubated with different concentrations of 5-FU (Figure 4A) or Dox (Figure 4B) in the absence (◆) or presence of BSO at the concentration of 5 $\mu\text{mol} \cdot \text{L}^{-1}$ (□), 50 $\mu\text{mol} \cdot \text{L}^{-1}$ (○) and 100 $\mu\text{mol} \cdot \text{L}^{-1}$ (■) respectively for 72 hr. Cell survival was determined by MTT assay. The values represent the means \pm SD of three experiments.

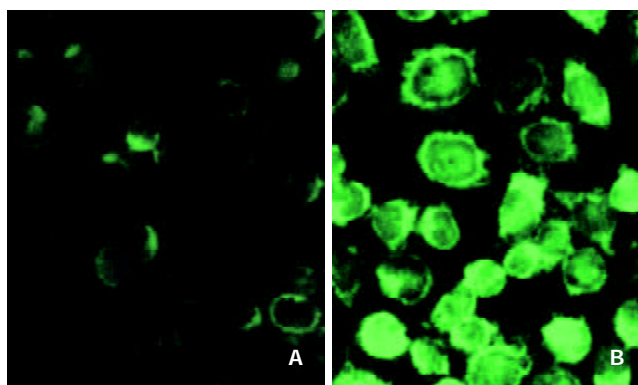


Figure 5 Immunocytochemical analysis of TS from parental and 5-FU resistant Bel₇₄₀₂ cells. The cytoprep slides were made as described in “Methods”. The slides were fixed in acetone, incubated with TS 106 and stained by using FITC-conjugated goat anti-mouse IgG. TS expression was determined by confocal laser scanning microscopy. (A) Bel₇₄₀₂ cells; (B) Bel₇₄₀₂/5-FU cells.

GSH levels and depletion of GSH by BSO

Since GSH implicates in the mechanism of MRP-mediated drug resistance, the intracellular GSH content was determined in Bel₇₄₀₂ and Bel₇₄₀₂/5-FU cells. As shown in Table 1, the GSH content in Bel₇₄₀₂/5-FU cells increased almost two folds in comparison with that in Bel₇₄₀₂ cells. When Bel₇₄₀₂/5-FU cells were incubated with BSO, an inhibitor of GSH biosynthesis, at 5, 50 and 100 $\mu\text{mol} \cdot \text{L}^{-1}$ for 24 hours, the GSH content in the cells decreased in a dose-dependent manner.

Table 1 GSH content and GSTs activity in Bel₇₄₀₂ and Bel₇₄₀₂/5-FU cells and the effect of BSO on the intracellular GSH content in Bel₇₄₀₂/5-FU cells

	BSO (μmol·L ⁻¹)	Subline	
		Bel ₇₄₀₂ /5-FU	Bel ₇₄₀₂
GSH content (mg/mg protein)	-	0.952±0.257	0.322±0.196 ^b
	5	0.520±0.107	
	50	0.427±0.200 ^a	
	100	0.403±0.135 ^a	
GSTs activity (nmol/min/mg protein)	-	19.1±1.9	14.8±1.2 ^c

GSH content was determined in Bel₇₄₀₂/5-FU cells treated with or without BSO for 24 hr and Bel₇₄₀₂ cells. GSTs activity was assayed by the method of Ghalia *et al*^[22] using 30 mmol·L⁻¹ GSH and 30 mmol·L⁻¹ CDNB as substrates. Results are presented as mean ±SD from three independent experiments, each performed in triplicates. Differences for significance were tested by using a Student's *t*-test: ^a*P*<0.05, ^b*P*<0.01 vs Bel₇₄₀₂/5-FU cells without the addition of BSO; ^c*P*<0.05 vs the GSTs activity in Bel₇₄₀₂/5-FU cells

Effect of BSO on the resistance of Bel₇₄₀₂/5-FU cells

Sublethal concentrations of BSO (5, 50, 100 μmol·L⁻¹) were added to test the role of GSH in the sensitivity of Bel₇₄₀₂/5-FU cells to 5-FU and Dox. As a result, the addition of BSO caused a slight left shift in the dose-effect pattern of 5-FU (Figure 4A) and Dox (Figure 4B) in Bel₇₄₀₂/5-FU cells, indicating that the inhibition of GSH biosynthesis by BSO only had a weak reversal activity on the resistance of Bel₇₄₀₂/5-FU cells.

GST enzymatic activity

GSTs catalyze the coupling reaction of intracellular electrophiles with GSH. Elevation of GSTs particularly GSTp is associated with an acquired resistance of cells to certain anticancer drugs. Table 1 showed the total GST activity in Bel₇₄₀₂/5-FU cells were 29.1 % higher than that in Bel₇₄₀₂ cells. The difference between both cell lines was significant (*P*<0.05).

TS levels

In order to determine whether there is difference of TS expression in Bel₇₄₀₂ and Bel₇₄₀₂/5-FU cell lines, an immunocytochemical analysis was performed by using MAb TS 106. As shown in Figure 5, the pattern of TS 106 reactivity was a dark granular appearance and diffusely spread throughout the cytoplasm in Bel₇₄₀₂/5-FU cells. The cytochemical staining intensity of TS 106 in Bel₇₄₀₂/5-FU cells (Figure 5B) markedly increased as compared with the parental Bel₇₄₀₂ cells (Figure 5A).

DISCUSSION

In vitro continuous stepwise exposure of HCC cell line Bel₇₄₀₂ to 5-FU for 6 months resulted in a 160 fold resistant subline Bel₇₄₀₂/5-FU in our laboratory. The established resistance to 5-FU only slightly decreased or remained unchanged without continuous exposure to 5-FU for one month. Verapamil, a classical MDR reversal agent, significantly reversed the resistance to VCR, taxol and Dox with similar fold reversal in Bel₇₄₀₂ and Bel₇₄₀₂/5-FU cell lines, but it only had little effect on the chemosensitivity to 5-FU and hydroxycamptothecine in both cells. In our previous study on the required resistant mechanism of Bel₇₄₀₂/5-FU cells to 5-FU, we found that there was no difference in the expression level of the *mdr-1* product P-gp between the two cells by immunocytochemical staining

and Western-blotting analysis.

Up-regulation of MRP protein was observed by using the monoclonal antibody against MRP, MRPr-1 in this study. It was reported that^[26] the gene expression of MRP correlated well with 5-FU resistance in 7 human gastrointestinal cancer cell lines. In some multidrug resistance cancer cells without alterations in levels of MRP, no resistance to 5-FU was found^[27,28]. MRP has been postulated to cause multidrug resistance by exporting the anticancer drugs out of the cells, then the accumulation of drugs in cells decreased. Unlike P-gp, MRP can be localized in both the plasma membrane and membranes of intracellular organelles, including the endoplasmic reticulum and Golgi apparatus, thus cause intracellular or cytoplasmic sequestration of drug and prevent pharmaceuticals from reaching their cellular targets^[25,29]. Anticancer drugs such as anthracyclines localize to the nucleus and cytoplasm with a high nuclear/cytoplasmic ratio in sensitive cancer cells. In contrast, anthracyclines accumulate mainly in the perinuclear region or in cytoplasmic vesicles in drug-selected MRP-overexpressing cells, and much less in the nucleus. In our experiments, the functional activity of MRP was investigated by flow cytometry using FLU accumulation as a probe. The accumulation of FLU in Bel₇₄₀₂/5-FU cells was only about half of the amount of FLU in the parental Bel₇₄₀₂ cells, suggesting an increased activity of MRP in the resistant Bel₇₄₀₂/5-FU cells. Furthermore, decreased nuclear/cytoplasmic ratio of FLU fluorescence was observed in Bel₇₄₀₂/5-FU cells as compared to that in the parental Bel₇₄₀₂ cells. All of these observations indicated that the decreased accumulation of FLU and the alteration in FLU distribution in Bel₇₄₀₂/5-FU cells may stem from up-regulated MRP.

MRP has been suggested to be the GS-X pump for multivalent organic anions such as cysteinyl leukotrienes, GSH disulfide and various GSH S-conjugates^[8,30]. Intracellular GSH content is closely related to MRP-mediated multidrug resistance, since GSH interacts directly with MRP and this interaction causes a change in MRP structure that facilitates the binding and/or transport of anticancer drugs. Alternatively, GSH and anticancer drugs may spontaneously form a complex that behaves as a MRP substrate^[31,32]. Using selective GSH modulators to decrease cellular GSH levels can sensitize tumor cells to the killing effects of chemotherapeutics^[33]. BSO is a specific inhibitor of the rate-limiting enzyme of the GSH biosynthetic pathway, γ-glutamylcysteine synthetase. Treatment of either drug-selected or transfected resistant cells with BSO markedly depletes GSH and restores sensitivity to anticancer drugs that are transported by MRP^[34-36]. In the present report, the resistant Bel₇₄₀₂/5-FU cells exhibited a significantly higher intracellular GSH content and higher overall GST activity than 5-FU sensitive Bel₇₄₀₂ cells. Addition of BSO dose-dependently reduced the GSH content in Bel₇₄₀₂/5-FU cells. However, only a weak enhancement in the cytotoxicity of 5-FU and Dox to Bel₇₄₀₂/5-FU cells by BSO was observed in growth inhibition assay. Moreover, BSO restores sensitivity to Dox more effectively than to 5-FU. Till now there is no direct evidence that 5-FU is a substrate of GSTs or MRP. It appeared that the elevated levels of the GSH/GST system and higher expression of MRP did not to play an important role in the 5-FU resistance development. These alterations maybe associated with the cross-resistance of Dox and cytarabine in the Bel₇₄₀₂/5-FU cells.

5-FU is widely used in the treatment of breast, gastrointestinal, and head and neck cancers. The induction of TS following short-term 5-FU exposure is a generally observed phenomenon in malignant breast and colon cells^[37]. A number of clinical

evaluations referred to the relationship of TS expression in tumors to clinical response and survival of cancer patients receiving 5-FU-based chemotherapy^[38,39]. Fukushima *et al*^[40] established a human tumor sub-line resistant to 5-FU by using colorectal xenografts in nude mice and an increase in TS activity was observed in this resistant cell. According to our study, the 5-FU resistant HCC cells exhibited the elevated levels of TS protein by immunocytochemical detection, which is a sensitive and quantitative method of detecting human TS by monoantibody TS 106 by a direct comparison of Western blotting and biochemical TS assay^[22]. Although it has been demonstrated a close correlation between TS protein levels and sensitivity to 5-FU in clinic, several other mechanisms of acquired resistance to 5-FU have been attributed to TS, such as gene amplification, increased dUMP levels, decreased FdUMP accumulation, decreased stability of ternary complex, depletion of intracellular folates and so on^[41]. Only TS protein expression was investigated in this research. Further investigations should be performed to determine whether other altered effects on TS occur in this Bel₇₄₀₂/5-FU cells.

In summary, our results clearly showed that TS expression increased remarkably in 5-FU resistant Bel₇₄₀₂/5-FU cells. In addition, the expression of MRP as well as GSTs activity and GSH content also increased. The overexpression of TS may be the main cause for the resistance development of 5-FU. The changes in MRP and GSH/GST system seemed to result in the cross-resistance of Dox in Bel₇₄₀₂/5-FU cells.

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